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Pathogenesis of bacillary dysentery in laboratory animals

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PROGRESS IN CLARIFYING the disease process of bacillary dysentery has been hampered by the lack of a convenient animal with which to work, for man, the chimpanzee, and the monkey are the only natural hosts. The result is that after 60-odd years of research, we do not have any notion of how pathogenic dysentery bacilli differ from nonpathogenic *Escherichia coli*. Indeed, the fundamental problem of defining the characteristics which endow the former with the capacity to cause dysentery while the latter passes through the bowel, usually without causing symptoms, has not been answered. We and others have attempted to study the infection in small laboratory animals in the hope that the findings obtained under these artificial conditions might give us some hints on the disease process as it occurs in nature. Much of the experimental work in the study of bacillary dysentery has been carried out in mice infected by the intraperitoneal route. However, an approach such as this is somewhat limited, for the factors influencing infection of the peritoneal cavity must necessarily differ from those operating in the intestinal tract.

While normal guinea pigs exhibit little or no reaction after oral administration of virulent dysentery bacilli followed by an intraperitoneal injection of opium, animals which have been deprived of food for 4 days usually die after being fed the bacteria, providing a drug such as opium is injected (7). It is this experimental model, the infected starved guinea pig, which we should like to characterize and try to relate the findings obtained from it to the natural infection. First, the model infection is specific in the sense that animals die following oral challenge with 1×10 *Shigella flexneri* but survive the feeding of even higher doses of *E. coli* strains isolated from healthy human beings (Table 1). In addition, living dysentery bacilli are required to cause a fatal infection: animals survive the oral administration of 10^{11} dead cells but succumb when 10^7 living bacteria are administered per os (Table 2).

Death usually occurs 24–48 hr after the challenge strain is fed but can take place as long as a week later. Diarrhea is not seen in animals dying within 48 hr of challenge but is common in the relatively few animals that succumb later in the disease process. The infection is limited to the bowel; bacteremia has not been observed. Ulcerative lesions of the bowel not unlike those observed in the natural host are noted. The distribution of these lesions depends upon the time when the animal succumbs. If it dies within 48 hr after challenge, lesions of the small intestine predominate (Fig. 1), whereas severe lesions of the cecum and colon are seen in those animals dying at a later time (Fig. 2). The intestinal tract of starved animals fed heat-killed bacteria remains normal. Similarly, the normal anatomical features of the intestinal tract are preserved when living cultures of *E. coli* or nonpathogenic strains of *Shigellae* are administered.

Many factors, no doubt, are involved in rendering the starved guinea pig susceptible to the fatal enteric infection with dysentery bacilli. We have studied two of these in some detail. First, histologic examination of the major organs of starved guinea pigs revealed that the only consistent change brought about by the starvation procedure was a centrilobular fatty degeneration of the liver. This striking change led us to try a common chemical, hepatotoxin, in an attempt to reproduce the same pathological changes. It was found that a subcutaneous injection of a sublethal dose of carbon tetrachloride could substitute for the starvation period. If one gave a small dose of carbon tetrachloride, 24–48 hr prior to oral challenge with dysentery bacilli, the pattern of death and pathological changes in the intestinal tract were the same as those seen in animals which had been starved (8).

These experiments suggested that a physiologic lesion of the liver played a role in the over-all reaction of the animal to the experimental infection. The liver has long been considered an organ of detoxification, and the results of toxicity experiments, summarized in Table 3, demonstrate that both starved and carbon tetrachloride-treated animals are much more susceptible than normal animals to bacterial endotoxin, the only known toxic product of *S. flexneri* (9). The lesion of the liver must be somewhat specific in that the administration of allyl alcohol, which also causes severe changes in the liver, does not appreciably increase the animals susceptibility to endotoxin (W. E. Farrar, Jr., personal communication). Others have demonstrated that liver extracts have the capacity to inactivate endotoxin in vitro and that the inactivation process appears to be enzymatic in nature

TABLE 1. *Susceptibility of starved* Hartley strain guinea pigs to the oral administration of strains of Enterobacteriaceae*

Strain	Challenge Dose	Deaths Total
S. flexneri 2a strain 2457	1×10^7	8/10
E. coli† HS	2×10^8	0/10
E. coli WS	2.2×10^8	0/10
E. coli EL	2.3×10^8	0/10

* Animals starved 4 days prior to challenge; 1 ml tincture of opium injected intraperitoneally after challenge. †*E. coli* strains isolated from healthy, adult human beings.

TABLE 2. *Susceptibility of starved* Hartley strain guinea pigs to the oral administration of heat-killed Shigella flexneri 2a*

Dose	Deaths/Total
Killed *S. flexneri* 2a	
2×10^{11} cells	0/10
2×10^{10} cells	1/10
2×10^9 cells	0/10
2×10^8 cells	0/10
Living *S. flexneri* 2a	
5×10^7 cells	8/10

* Animals starved 4 days prior to challenge; 1 ml tincture of opium injected intraperitoneally after challenge.

FIG. 1. Terminal ileum of starved guinea pig 20 hr after oral challenge with virulent *Shigella flexneri* 2a. There is an acute inflammatory reaction in the lamina propria, and the villus architecture is greatly altered. Hemotoxylin-eosin stain. × 91.

(15). More recently, Farrar and Magnani (3) have been able to demonstrate that liver preparations from carbon tetrachloride-treated guinea pigs have a markedly reduced capacity to inactivate endotoxin in vitro than do similar extracts of liver from normal animals. In addition, evidence has been presented which indicates that the enzyme(s) involved in this inactivation process have to do with fatty acid oxidation (1).

A second factor which has been studied in this experimental model is the small intestine which has been found to be intimately involved in the infection which results in the death of the majority of the modified animals (6). Most of the animals which succumb do so 24–48 hr after challenge and dysentery bacilli in large numbers are isolated from the proximal small bowel. Furthermore, if *S. flexneri* cells are injected into the duodenum the animal dies, but animals usually survive comparable challenges inoculated into the cecum. On the other hand, in the relatively few animals which exhibit symptoms of diarrhea and which succumb at times later than 72 hr post-challenge, the colon is predominately involved. Classical bacillary dysentery is considered to represent an infection of the large intestine. However, it is possible in some situations that the small intestine is the major area which is affected, and in these cases the over-all picture of the disease might be expected to be quite different from that which is commonly seen. Indeed organisms of the family *Enterobacteriaceae* are not normal residents of the small intestine, and it has been suggested that even nonpathogens multiplying in this region for short periods might cause transient changes in the mucosal architecture and mild symptoms (2). The major defense mechanism thus far found for maintaining the small intestine relatively free of gram-negative microorganisms is its motility (2, 6); thus in the experimental model, this had to be blocked by the injection of a drug such as opium so that

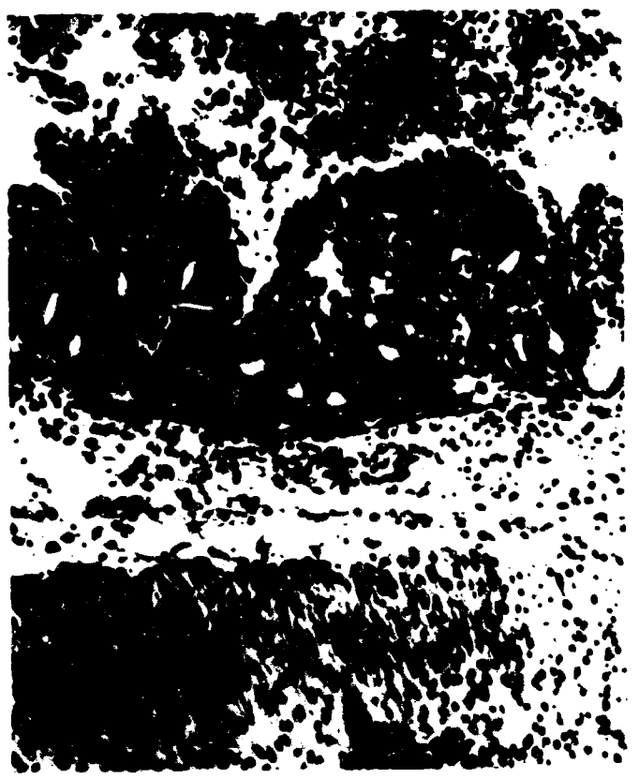

FIG. 2. Colon of starved guinea pig 72 hr after oral challenge with virulent *S. flexneri* 2a. There is an acute inflammatory reaction in the mucosa, and the epithelium is virtually destroyed. Remnants of crypt glands may be seen. There is an inflammatory exudate in the lumen. Hemotoxylin-eosin stain. × 126.

TABLE 3. *LD_{50} Of bacterial endotoxin* for normal, starved and carbontetrachloride-treated Hartley strain guinea pigs*

	Treatment		
	Normal	Starved†	Carbon tetrachloride‡
LD_{50} (μg)	2,500	52.8	4.0
SE	1,766	26.4	3.4

* Endotoxin injected iv in 0.5 ml vol. †Animals starved 4 days prior to the injection of endotoxin. ‡0.15 ml CCl_4 injected subcutaneously 30 hr prior to the injection of endotoxin.

the process of a severe infection could proceed resulting in the acute death of the animal.

The experimental model, then, consists of an acute infection of the small intestine in the animal which at the same time has been rendered quite sensitive to the toxic products of the infecting organism. This is obviously not classical bacillary dysentery which at least in young adults is not usually fatal. However, the animal model bears a similarity to the Ekiri syndrome which in the past has been quite common in Japanese children (16). This syndrome appears to be a fulminating enteric infection, usually with *Shigella*, but can also result from infection with strains of pathogenic *E. coli* or *Salmonellae*. Diarrhea may or may not occur. The child appears to be in a state of shock, and convulsions may or may not be present. In extreme cases, death may occur within 8 hr after the onset of symptoms. Ekiri is not limited to Japan, however, for individuals with similar symptoms have been seen in the United States (10, 11). The small intestine may be as important a factor in the Ekiri syndrome as it is in the experimental guinea pig model. In addition, the extreme sensitivity of the child to the infection may be due in large part to the child's inability to handle the toxic products of the infecting organism and not necessarily to a unique sensitivity of the target organs to small amounts of the bacterial toxins.

There is no doubt that host factors play a major role in the course of the disease after infection has taken place. It is equally certain that the ability to cause disease resides in the infecting organism. In order to understand the attributes which a virulent dysentery bacillus must possess to be pathogenic, we have compared a virulent parent dysentery strain with a mutant strain, derived from it, whose capacity to cause disease in the experimental animal is completely altered. When the parent strain was fed to starved guinea pigs, 90% succumbed; on the other hand, all of the animals survived similar challenge doses with the mutant (14). Not only was the mutant strain unable to kill, it did not even cause histologic changes in the bowel (Fig. 3 and 4). Indeed, the animal reacted no differently to oral challenge with the mutant dysentery strain than with *E. coli*. These observations made in the experimental guinea pig model were extended to a natural host, the monkey (13). Approximately 35% of monkeys fed 5×10^{10} cells of the parent strain exhibited symptoms ranging from diarrhea to classical dysentery. On the other hand, animals receiving the mutant strain remained normal.

Studies were conducted in an attempt to define the attributes which are responsible for the pathogenicity of the parent strain for starved guinea pigs and monkeys and

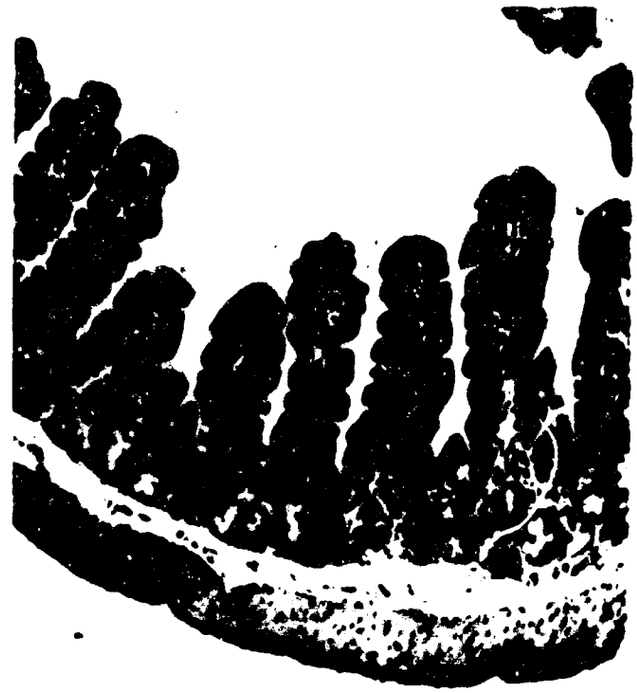

FIG. 3. Terminal ileum of guinea pig 20 hr after oral challenge with avirulent mutant strain of *S. flexneri* 2a. Epithelium is intact. Lamina propria is normal, and villus architecture unaltered. Compare with Fig. 1. Hemotoxylin-eosin stain. X 91.

FIG. 4. Colon of guinea pig 48 hr after oral challenge with avirulent mutant strain of *S. flexneri* 2a. Epithelium is intact. There is no evidence of an inflammatory reaction. Compare with Fig. 2. Hemotoxylin-eosin stain X 228.

FIG. 5. Frozen section of ileum from a starved guinea pig 8 hr after oral challenge with the virulent parent strain of *S. flexneri* 2a. Section was treated with fluorescein-labeled rabbit anti-*S. flexneri* 2a globulin. Arrows point to some of the fluorescing *Shigellae* in lamina propria (L) of two villi. *Shigellae* phagocytosed by an inflammatory cell can be seen at (a). (IL) denotes the intestinal lumen. Original magnification, × 240.

which are absent or masked in the mutant strain. Both strains are serologically identical when tested by agglutinin adsorption procedures and no difference has been noted in their reactions when examined by the Ouchterlony immunodiffusion technique. Both strains kill mice equally well when suspended in 5% hog gastric mucin and injected intraperitoneally. The LD_{50} for mice ranges between 1×10^1 to 1×10^4 cells depending on the strain of mice employed. Thus, here then is an obvious discrepancy in the criteria used to assess pathogenicity, for only the parent strain is capable of causing symptoms when fed to either the starved guinea pig or the monkey. Both strains are equally toxic for mice when suspensions of acetone-killed and dried cells are injected intraperitoneally; the LD_{50} dose is approximately 8 mg. Both strains grow equally well in vitro and also, as far as we can tell, in vivo (14). Thus, both strains appear to be identical except for the fact that the parent produces disease while the mutant does not.

Study of the infection with the fluorescent antibody technique led not only to an explanation for the difference in the pathogenicity of the two strains but also to an insight into the mechanism of ulcer formation. The previous concept of the process of ulcer formation envisioned consecutive waves of absorption and excretion of heat-stable toxin across the intestinal wall resulting in hypoxia and death of the epithelium (4). It is, of course, true that the intravenous injection of heat-killed gram-negative organisms or isolated endotoxin results in bowel pathology in some animal species. However, neither symptoms nor significant alteration of the intestinal mucosa are observed following repeated oral administration of large doses of these materials. Furthermore, if toxin absorption from the intestinal lumen is an essential feature of the disease process, one would expect pathogenic *Shigella* to produce a toxin or toxins which nonpathogenic *E. coli* fail to elaborate. With the exception of neurotoxin of *S. dysenteriae* 1, differences in toxin production by strains of *Shigella* on one hand and *E. coli* on the other have never been demonstrated. Thus, an alternate explanation of the mechanism of ulcer formation appears to be in order.

LaBrec and Formal (12), using the fluorescent antibody technique, have observed that virulent dysentery bacilli have the capacity to cross the intestinal epithelial barrier and enter the lamina propria (Fig. 5). Recent studies (13) indicate that the organisms reach the lamina propria by penetrating and passing through the intestinal epithelial cell (Fig. 6). This is an attribute of the parental virulent strain; it is not a characteristic possessed by the avirulent variant. When fed to animals, the avirulent variant remains in the lumen of the intestine,

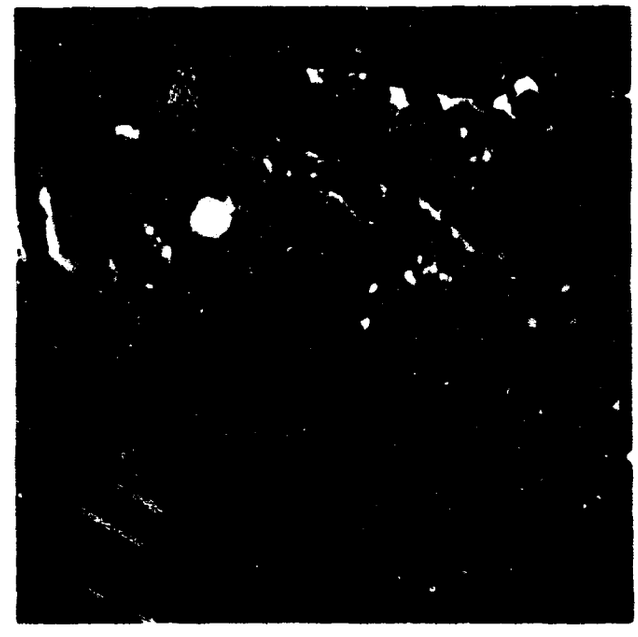

FIG. 6. Frozen section of terminal ileum from a starved guinea pig 8 hr after oral challenge with virulent strain of *S. flexneri* 2a. Section was treated with fluorescein-labeled rabbit anti-*S. flexneri* 2a globulin. Specifically fluorescing *Shigellae* (arrows) can be seen within epithelial cells. The organisms at (a) are phagocytosed. (L) denotes the lamina propria of a single villus. (C) is the crypt area. Original magnification, × 440.

and even though it may be in contact with the epithelium, it has not been observed to enter the epithelial cells or the lamina propria (Fig. 7). This capacity to enter the mucosal tissue via the epithelial cell is the only property so far detected which distinguishes the virulent from the nonvirulent strain.

On the basis of these observations, one may then construct a chain of events which results in the formation of an ulcerative lesion of the intestinal tract. First, the virulent dysentery bacilli enter the epithelial cell where they may multiply to some extent (Fig. 6), from here they enter the lamina propria (Fig. 5). Survival and multiplication of the organisms in this site leads to an accumulation of metabolic products and the local release of endotoxin; these cause hypoxia and death of the epithelium, resulting in an ulcer (Fig. 8). This process continues until the defense mechanisms of the host become sufficient to prevent the pathogen from further invasion of the bowel wall.

If this thesis is correct, one need not postulate the absorption of toxic materials from the bowel lumen; rather, these are released in the mucosal tissue by the pathogenic organisms which have penetrated to this site. Moreover, it follows from the hypothesis that epithelial cell penetration and at least limited survival in the lamina propria are the necessary properties conferring on the dysentery bacilli the capacity to produce disease. If this is the case, then it is these characteristics rather than some undetected difference in toxin production which serves to set the dysentery bacilli apart from the nonpathogenic *E. coli*, and avirulent *Shigellae*.

FIG. 7. Frozen section of ileum from a starved guinea pig 8 hr after oral challenge with avirulent mutant strain of *S. flexneri* 2a. Section was treated with fluorescein-labeled rabbit anti-*S. flexneri* globulin. Many fluorescing *Shigellae* are seen in intestinal lumen (IL). No fluorescing organisms can be seen in the mucosal epithelium or in the lamina propria (L). The histiocytes (h) in lamina propria contain inclusions which have a primary fluorescence of yellow or orange color. (Original magnification, × 240.

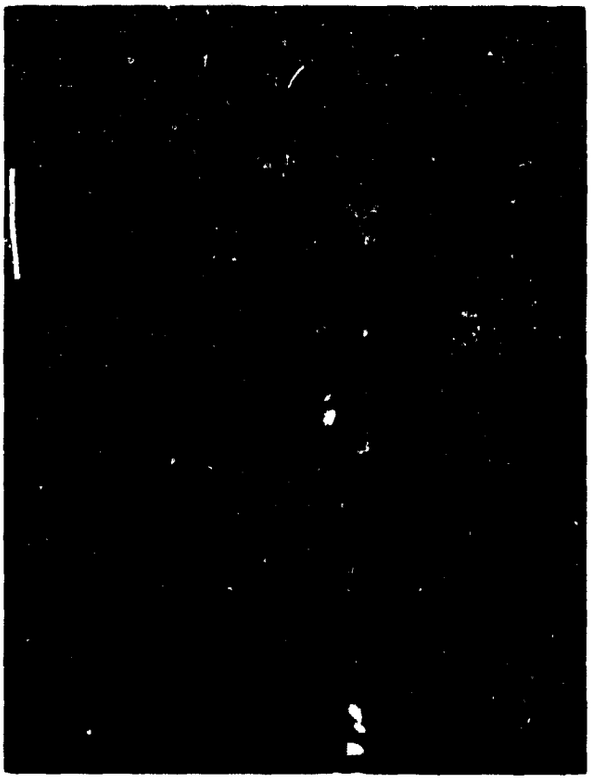

FIG. 8. Frozen section of ileum from a starved guinea pig 24 hr after oral challenge with the virulent parent strain of *S. flexneri* 2a. The section was treated with fluorescein-labeled rabbit anti-*S. flexneri* 2a globulin. The normal mucosal architecture is obscure. The intestinal lumen (IL) and the ulcerated portion of the mucosa (mu) contain inflammatory cells, tissue debris, and fluorescing *Shigellae*. (e) is the mucosal epithelium. Original magnification, × 130.

The severity of symptoms experienced by an individual could depend upon the number of sites on the mucosa which the pathogen penetrates, the ability of the organism to multiply in the lamina propria, the sensitivity of the host to the toxic products of the invading organism, and the rapidity of response of the defense mechanisms of the host to the infection. The experimental model just described offers opportunity to gain insight into some of these possibilities. The possible role of the liver and small intestine in enteric infections has already been mentioned. At present studies are being carried out with certain *E. coli Shigella* hybrids (5) which have the capacity to penetrate the intestinal epithelium but lack the ability to multiply in the lamina propria. While alteration in bowel histology is observed, the starved guinea pig invariably survives infection with such hybrid strains (unpublished observations). It is hoped that such studies will also yield some clues concerning the defense mechanisms of the bowel wall.

The concepts expressed here were formulated exclusively from observations made in an artificial experimental infection in the guinea pig. While they are of interest, they can be of little or no practical value unless they re-

flect the disease process as it occurs in nature. In so far as we have been able to test them, e.g., the role of bowel motility as a defense mechanism, the capacity of mutants to cause disease, and the penetration of mucosal epithelial cells as a step in the process of ulcer formation, the same concepts also hold for one natural host, the monkey. It is expected that these can be extended to another natural host—man.

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